

# The MDM-2 Antagonist Nutlin-3 Promotes the Maturation of Acute Myeloid Leukemic Blasts<sup>1</sup>

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## Abstract

The small-molecule inhibitor of murine double minute (MDM-2), Nutlin-3, induced variable apoptosis in primary acute myeloid leukemia (AML) blasts and promoted myeloid maturation of surviving cells, as demonstrated by analysis of CD11b and CD14 surface antigens and by morphologic examination. Although the best-characterized activity of Nutlin-3 is activation of the p53 pathway, Nutlin-3 induced maturation also in one AML sample characterized by p53 deletion, as well as in the p53<sup>-/-</sup> human myeloblastic HL-60 cell line. At the molecular level, the maturational activity of Nutlin-3 in HL-60 cells was accompanied by the induction of E2F1 transcription factor, and it was significantly counteracted by specific gene knockdown with small interfering RNA for E2F1. Moreover, Nutlin-3, as well as tumor necrosis factor (TNF)  $\alpha$ , potentiated the maturational activity of recombinant TNF-related apoptosis-inducing ligand (TRAIL) in HL-60 cells. However, although TNF- $\alpha$  significantly counteracted the proapoptotic activity of TRAIL, Nutlin-3 did not interfere with the proapoptotic activity of TRAIL. Taken together, these data disclose a novel, potentially relevant therapeutic role for Nutlin-3 in the treatment of both p53 wild-type and p53<sup>-/-</sup> AML, possibly in association with recombinant TRAIL.

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that besides interacting with p53, the *mdm2* gene encodes a protein consisting of several domains with different functions: 1) an N-terminal domain that contains binding sites for the transcription factors p53 and E2F1; 2) an acidic domain that interacts with the tumor suppressor p14ARF; 3) a putative Zn finger and a binding site for the retinoblastoma protein Rb; and 4) a RING finger and an E3 ligase domain that are responsible for the ubiquitination of p53 [2,3].

Recently, Nutlin-3, a small-molecule inhibitor of MDM-2, has been developed [4]. Nutlin-3 binds to MDM-2, releasing p53 from negative control and leading to effective p53 stabilization and activation in cells with wild-type, but not mutant or deleted, p53 [4,5]. Of note, recent studies have demonstrated that Nutlin-3, used alone or in combination with chemotherapeutic drugs, effectively increases the degree of apoptosis in acute myeloid leukemia (AML), as well as in other hematologic malignancies characterized by wild-type p53 [6–9].

Although the therapeutic effect of most conventional anti-cancer drugs has been attributed for years to their ability to induce apoptosis, it has been recognized that growth arrest with morphologic features reminiscent of terminal maturation constitutes an alternative drug-induced response program controlled, at least in part, by the p53 pathway [10]. Thus, the experiments illustrated in this study were designed to investigate whether Nutlin-3, used alone or in combination with the death-inducing ligand TNF-related apoptosis-inducing ligand (TRAIL), was able to modulate the maturation of primary blasts obtained by AML patients. Moreover, we have performed a series of experiments in the p53<sup>-/-</sup> HL-60 myeloblastic leukemia cell line, which can be induced to undergo monocytic/granulocytic terminal differentiation by a variety of chemical and biologic agents [11,12], and based on recent data demonstrating

## Introduction

The human homologue of murine double minute (MDM-2) is an oncogene overexpressed in different types of malignancies, including leukemias (reviewed in Bueso-Ramos et al. [1]), which plays an important role in cancer development and progression. The best-characterized biologic activity of MDM-2 is to antagonize the transcriptional activity of p53 and to target p53 for ubiquitin-dependent proteolysis [2]. The *mdm2* gene is itself transcriptionally activated by p53 in a regulatory feedback loop [3]. It is noteworthy, however,

Abbreviations: MDM-2, murine double minute; PI, propidium iodide; siRNA, small interfering RNA; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; TRAIL, TNF-related apoptosis-inducing ligand

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that Nutlin-3 is biologically active in both *p53* wild-type and *p53*-deleted or *p53*-mutated cells [13].

## Materials and Methods

### Patients and Cell Lines

Peripheral blood samples from 10 AML patients were collected in heparin-coated tubes (Table 1) following their provision of informed consent, in agreement with institutional guidelines. The diagnosis of AML was made by peripheral blood and bone marrow morphology and immune phenotyping. These patients had been without prior therapy. Peripheral blood mononuclear cells from AML patients were isolated by gradient centrifugation with a lymphocyte cell separation medium (Cedarlane Laboratories, Hornby, Ontario), and freshly purified AML blasts were used immediately after purification for different *in vitro* treatments. The AML patients' cells, as well as the *p53*<sup>-/-</sup> HL-60 and *p53* wild-type SKW6.4 cell lines, were cultured in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco) and seeded at an optimal cell density of  $0.8 \times 10^6$  to  $1.0 \times 10^6$  cells/ml before treatments.

Primary AML blasts or HL-60 cells were treated with predetermined optimal concentrations of Nutlin-3 (10  $\mu$ M; Sigma Aldrich, St. Louis, MO); recombinant TRAIL (0.1  $\mu$ g/ml), prepared as previously described [14]; or recombinant tumor necrosis factor (TNF)  $\alpha$  (10 ng/ml; R&D Systems, Minneapolis, MIN), used either alone or in combination.

### Assessment of Apoptosis and Cell Differentiation

At different times (1–3 days) posttreatment with Nutlin-3, TRAIL, or TNF- $\alpha$ , both cytotoxicity and maturation-inducing activity were assessed. In particular, samples were analyzed by: 1) counting the total number of viable cells by trypan blue dye exclusion; 2) evaluating the degree of apoptosis by Annexin V/propidium iodide (PI) double staining and flow cytometry and/or poly(ADP)ribose polymerase (PARP) cleavage in Western blot analysis; as previously described [15]; 3) monitoring the cell surface expression of CD14 and CD11b myeloid antigens [16,17]; and 4) examining the morphology of the cells by staining with May-Grunwald-Giemsa solution followed by light microscopic examination.

**Table 1.** Clinical and Laboratory Features of AML Patients.

Patient No.	AML	p53	% Viable Cells + Nutlin-3*
1	M4	Wild type	67
2	M4/M5	Wild type	73
3	M5	Wild type	51
4	M5	Wild type	77
5	M4/M5	Deleted	93
6	M5	Wild type	23
7	M5	Wild type	58
8	M5	Wild type	54
9	M5	Wild type	60
10	M5	Wild type	55

\*The percentage of viability was measured by trypan blue at 72 hours of treatment.

In particular, for flow cytometry analyses, both Annexin V–fluorescein isothiocyanate (FITC)/PI apoptosis kit and CD11b and CD14 phycoerythrin-conjugated or FITC-conjugated antibodies (Abs) were purchased from Immunotech (Marseille, France). Nonspecific fluorescence was assessed by using isotype-matched controls.

Flow cytometry analysis was also used to determine the cell-cycle profile. HL-60 cells were incubated with 50  $\mu$ M BrdU (Sigma Aldrich) at 37°C for 1 hour. Anti-BrdU was bound to BrdU incorporated into neosynthesized DNA, and the complex was detected by FITC-conjugated secondary Ab. Cells were stained with PI (50  $\mu$ g/ml) and analyzed by flow cytometry. To avoid nonspecific fluorescence from dead cells, live cells were gated tightly using forward scatter and side scatter. All flow cytometry analyses were performed by using a FACScan flow cytometer and Lysis II software (Becton Dickinson, San Jose, CA).

### Western Blot Analysis and Immunoprecipitation

Cells were lysed in a buffer containing 50 mM Tris–HCl pH 8.0, 250 mM NaCl, 0.5% NP-40, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 2  $\mu$ g/ml pepstatin, as described [18]. For Western blot analysis, 50 to 70  $\mu$ g of protein was resolved on SDS polyacrylamide gels and transferred to nitrocellulose membranes. Blots were incubated with monoclonal Abs anti-MDM-2, anti-p53, anti-DNA repair enzyme PARP (all from Santa Cruz Biotechnology, Santa Cruz, CA), anti-Rb protein (Becton Dickinson), or anti-tubulin (Sigma Aldrich) used for loading control. Membranes were washed and further incubated for 1 hour at room temperature with peroxidase-conjugated secondary Ab (Sigma Aldrich). Detection was then performed using the Renaissance chemiluminescent ECL kit (NEN Dupont, Boston, MA).

E2F1 was detected by immunoprecipitation experiments. For this purpose, at least  $5 \times 10^6$  to  $10 \times 10^6$  cells/sample were lysed in a buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20, 0.5 mM EDTA, and the protease inhibitor cocktail P8340 (Sigma Aldrich). Cell lysates were incubated with a polyclonal E2F1 Ab (Santa Cruz Biotechnology) for 12 hours at 4°C before the addition of 40  $\mu$ l of A/G Sepharose beads (Santa Cruz Biotechnology) for an additional 3 hours. Immunocomplex was washed thrice with lysis buffer, loaded on 10% PAGE gels, and detected by immunoblotting, as described above. In parallel, for each experiment, equal fractions (5–10%) of cell lysates from untreated and Nutlin-treated cultures were analyzed by Western blot analysis for tubulin levels, serving as controls of immunoprecipitation inputs.

Protein levels were densitometrically analyzed by the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Multiple film exposures were used to verify the linearity of the samples analyzed and to avoid saturation of the film.

### Transfection Experiments

HL-60 cells ( $1.2 \times 10^6$  cells/0.5 ml) were mixed with 1  $\mu$ g of enhanced green fluorescence protein (EGFP) plasmid or 2  $\mu$ g of small interfering RNA (siRNA) cocktails, transferred

to a 2.0-mm electroporation cuvette, and nucleofected with the Nucleofector Kit V (Amaxa, Cologne, Germany) using the program T-019 of the Nucleofector device (Amaxa Nucleofector II apparatus). After electroporation, cells were immediately transferred to a complete RPMI supplemented with 10% fetal calf serum and cultured at 37°C until analysis. Transfection efficiency was monitored in each experiment by scoring the percentage of EGFP-positive cells by flow cytometry analysis.

siRNA were designed and manufactured by Ambion, Inc. (Austin, TX) according to the method's current guidelines for effective knockdown. The following siRNA were tested: target 1 for *E2F1*, 5'-CCUGAUGAAUAUCUGUACUtt-3' (sense) and 5'-AGUACAGAUUAUCAUCAGGtg-3' (antisense); target 2 for *E2F1*, 5'-GGCCCGAUCGAUGUUUUCctt-3' (sense) and 5'-GGAAAACAUCGAUCGGGCCctt-3' (antisense).

Ambion's *Silencer* Negative Control siRNA were used to demonstrate that the transfection did not induce nonspecific effects on gene expression. A cocktail of two different negative control siRNA, each composed of a 19-bp scrambled sequence with 3' dT overhangs, was used. The sequences have no significant homology to any known gene sequence from humans and have been previously tested for lack of nonspecific effects on gene expression (Ambion).

#### Statistical Analysis

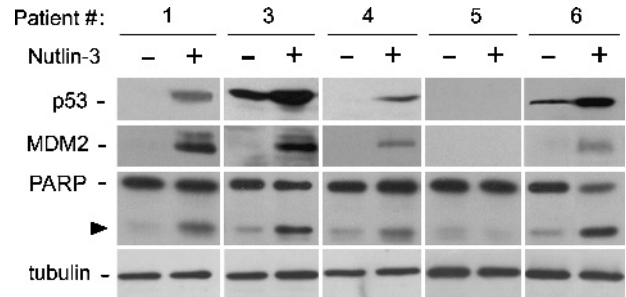
The results were evaluated using analysis of variance, with subsequent comparisons by Student's *t* test and Mann-Whitney rank-sum test. Statistical significance was defined as  $P < .05$ .

## Results

### Nutlin-3 Induces Variable Levels of Cytotoxicity in Primary AML Blasts

To investigate the effect of Nutlin-3 on cell viability, AML samples freshly isolated from patients free of therapy were incubated *in vitro* with predetermined optimal concentrations of Nutlin-3 (10  $\mu$ M) and were assayed for viability by trypan blue dye exclusion at 72 hours of treatment (Table 1). During this time frame, the number of viable cells in untreated cultures remained relatively constant, never dropping below 70% compared to the cell number seeded at time 0 ( $1 \times 10^6$  cells/ml). On Nutlin-3 treatment, a variable decrease in cell viability ( $61 \pm 18.5$ ; mean  $\pm$  SD of the percentage of cell viability compared with untreated controls; Table 1) was observed, with all patient samples being susceptible to Nutlin-3 cytotoxicity, with the exception of patient 5. The decrease in cell viability induced by Nutlin-3 was paralleled by a concomitant increase in the percentage of apoptosis, evaluated in terms of Annexin V-positive cells (data not shown) and/or PARP cleavage (Figure 1) with respect to control cultures.

As expected based on previous studies [4–9], on Western blot analysis, Nutlin-3, efficiently, although variably, upregulated p53 protein levels, as well as MDM-2, in all AML samples, with the exception of patient 5 (Figure 1). The lack



**Figure 1.** p53 and MDM-2 induction in response to Nutlin-3 in primary AML cells. AML cells were left untreated or were incubated with Nutlin-3 (10  $\mu$ M) before Western blot analysis. Reported here are representative samples (samples 1, 3, 4, and 6) showing a variable induction of p53 and MDM-2, and one sample (sample 5) showing a lack of induction of p53 and MDM-2. PARP cleavage (arrowhead; 80-kDa cleaved form) was also analyzed as an apoptotic marker. Tubulin staining is shown as loading control.

of induction of p53 in response to Nutlin-3 observed in patient 5 (Figure 1) has been previously shown to be a characteristic feature of tumor samples wherein p53 was deleted [4–9].

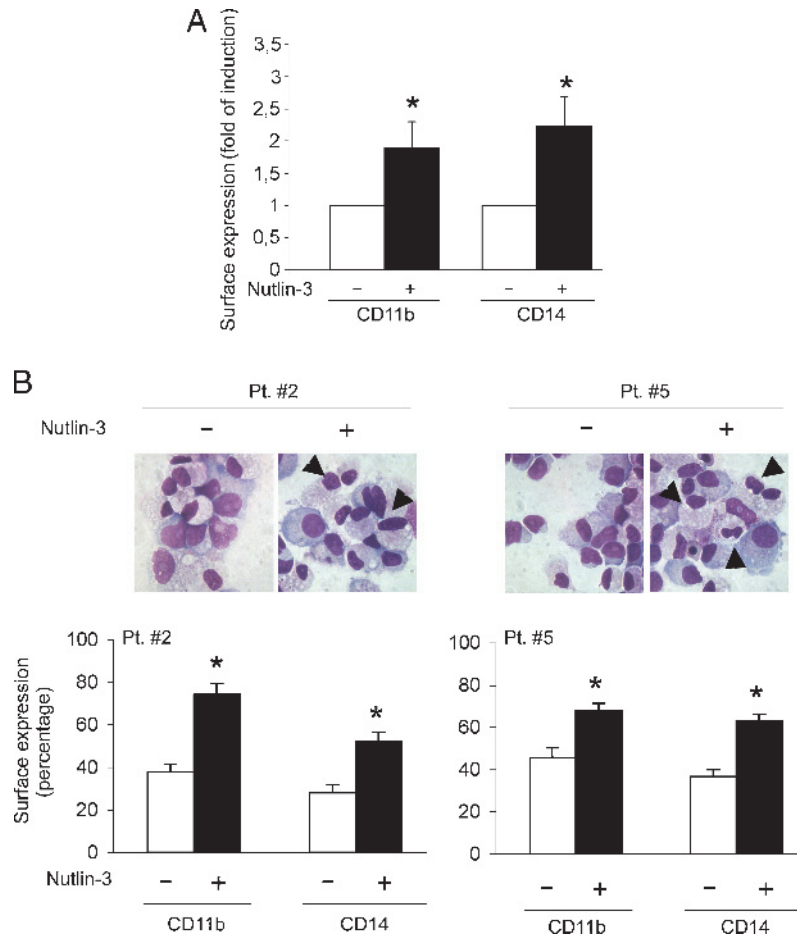
### Nutlin-3 Promotes Myeloid Maturation in Primary AML Blasts Independently of Its Proapoptotic Activity

Because an alternative therapeutic strategy to the induction of apoptosis is to promote terminal growth arrest and maturation of AML blasts, in the next experiments, we have investigated whether Nutlin-3 was able to modulate the phenotypic profile and morphology of AML cells that survived Nutlin-3 cytotoxicity. Independently of the basal expression of CD11b and CD14 antigens that was variable from sample to sample (data not shown), Nutlin-3 treatment showed a significant ( $P < .05$ ) induction of both surface CD11b and CD14 antigens at 72 hours (Figure 2A). It should be noticed that CD14 represents an excellent marker of monocytic maturation because it is undetectable on the surface of monocyte precursors and increases dramatically during their differentiation into monocytes [16]. Surface CD11b, particularly the  $\beta_2$  integrin CR3, is another classic marker of myeloid differentiation that is involved in cell adhesion and also functions as a complement receptor [17].

Flow cytometry results were confirmed by morphologic analyses, which showed an increment of cells with monocytic features following Nutlin-3 treatment (Figure 2B). It is particularly noteworthy that the ability of Nutlin-3 to promote myeloid maturation occurred also in the p53-deleted patient sample (patient 5; Figure 2B), indicating that the promaturation effect of Nutlin-3 was totally unrelated to its ability to induce apoptotic cell death.

### Nutlin-3 Promotes Maturation of the p53<sup>-/-</sup> Myeloblastic HL-60 Cell Line in an E2F1-Dependent Manner

The data obtained on primary AML blast samples suggested that Nutlin-3 might affect myeloid maturation not only in p53 wild-type cells but also in p53<sup>-/-</sup> cells. To further ascertain whether this biologic activity of Nutlin-3 occurred independently of p53, the next experiments were performed on the p53<sup>-/-</sup> AML HL-60 cell line (Figure 3A). Nutlin-3 alone did not induce a significant increase in apoptosis in HL-60



**Figure 2.** Maturation effect of Nutlin-3 on AML cells. AML cells were left untreated or were incubated with Nutlin-3 (10  $\mu$ M) for 72 hours and then analyzed for the surface expression of CD11b and CD14 myeloid antigens, by flow cytometry analysis, and for cell morphology, by microscopic examination. In (A), CD11b and CD14 expression in Nutlin-3-treated cultures was calculated as fold induction with respect to untreated control cultures, which were set to 1. Data are expressed as the mean  $\pm$  SD of the results obtained from all patients reported in Table 1. \* $P < .05$ . In (B), the effects of Nutlin-3 on the cell morphology and surface expression of CD11b and CD14 in two representative (sample 2, p53 wild-type cells; sample 5, p53-deleted cells) AML patient samples are shown. Cytoцентрифугed AML samples were stained with May-Grunwald-Giemsa solution. Arrowheads identify AML cells with morphologic features of more mature cells (original magnification,  $\times 40$ ). CD11b and CD14 surface expression is reported as the percentage of positive cells, and data are expressed as the mean  $\pm$  SD of three independent analyses. \* $P < .05$ .

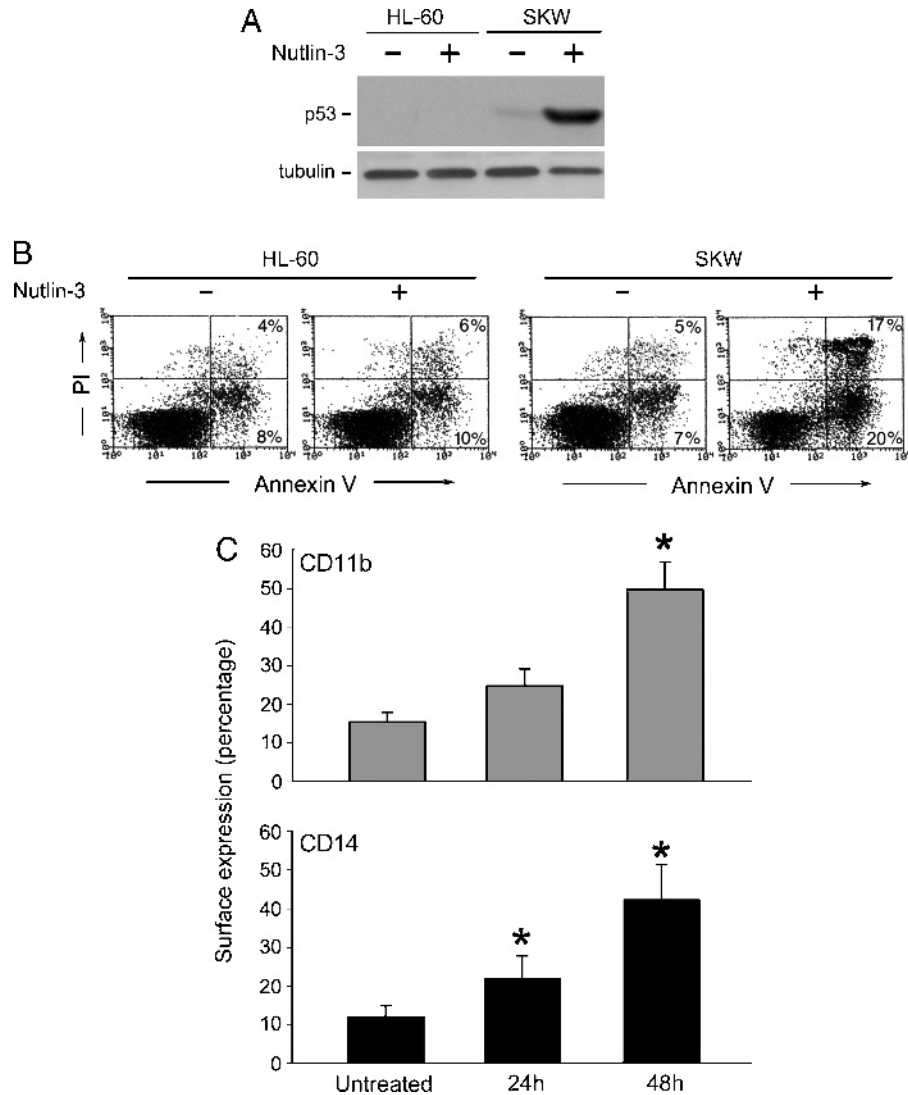
cells (Figure 3B), in keeping with a central role of the p53 pathway in mediating the proapoptotic activity of Nutlin-3 [4–9]. In spite of the lack of induction of cytotoxicity, treatment with Nutlin-3 induced a progressive increase in the surface expression of both CD11b and CD14 in p53<sup>-/-</sup> HL-60 cells (Figure 3C). Moreover, the ability of Nutlin-3 to upregulate the surface levels of CD11b and CD14 in HL-60 was more evident than in primary AML blasts, suggesting that this cell line is a good model for the study of the maturational effects of Nutlin-3.

Because previous studies have shown that differentiation of HL-60 cells was accompanied by exit from the cell cycle [11,12,16,17,19], the next experiments were performed to investigate whether Nutlin-3 induced changes in the cell-cycle distribution of HL-60 cells. As shown in Figure 4A, Nutlin-3 induced a significant ( $P < .05$ ) accumulation of HL-60 in the G<sub>2</sub>/M phase of the cell cycle. The ability of Nutlin-3 to promote the accumulation of HL-60 cells in the G<sub>2</sub>/M phase was markedly different from the biologic activity of other compounds, such as the natural compound

saucernetin-7, which promoted cell-cycle arrest in G<sub>0</sub>/G<sub>1</sub> coupled to the monocytic maturation of HL-60 cells [19]. Because MDM-2 has been shown to modulate the levels of Rb protein [20,21], the levels of this transcription factor were analyzed by Western blot analysis (Figure 4B). At variance with other maturational compounds, which induced a progressive increase in the hypophosphorylated form of Rb protein [19], Nutlin-3 decreased the total levels of Rb protein in HL-60 cells (Figure 4B).

Besides interacting with p53 and Rb protein, MDM-2 also interacts with other transcription factors, which play an important role in the regulation of cell-cycle progression and maturation [2,3]. In particular, it has been recently shown that Nutlin-3 induced the expression of E2F1 in cell lines derived from solid tumors [13]. Therefore, we next investigated the effect of Nutlin-3 on E2F1 expression in HL-60 cells. Because the amount of E2F1 in HL-60 and AML patient cell lysates was too low to be detected by Western blot analysis on whole-cell lysates (data not shown), immunoprecipitation assay was performed. As shown in Figure 4C,





**Figure 3.** Maturation effect of Nutlin-3 on HL-60 cells. HL-60 cells were left untreated or were incubated with Nutlin-3 (10  $\mu$ M). (A) Western blot analysis of cell lysates shows lack of p53 accumulation in HL-60 in response to Nutlin-3. Tubulin staining is reported as loading control. (B) Flow cytometry profiles after Annexin V/PI staining show lack of apoptosis induction in response to Nutlin-3 in HL-60 cultures. For both Western blot analysis (A) and flow cytometry (B) experiments, p53 wild-type SKW cells were analyzed in parallel as positive control. Results representative of three separate experiments are shown. (C) The time-dependent effect of Nutlin-3 (10  $\mu$ M) on the surface expression of CD11b and CD14 on HL-60 cells was quantitatively evaluated by flow cytometry. Data are presented as the mean  $\pm$  SD of five independent experiments performed in duplicate. \* $P < .05$ .

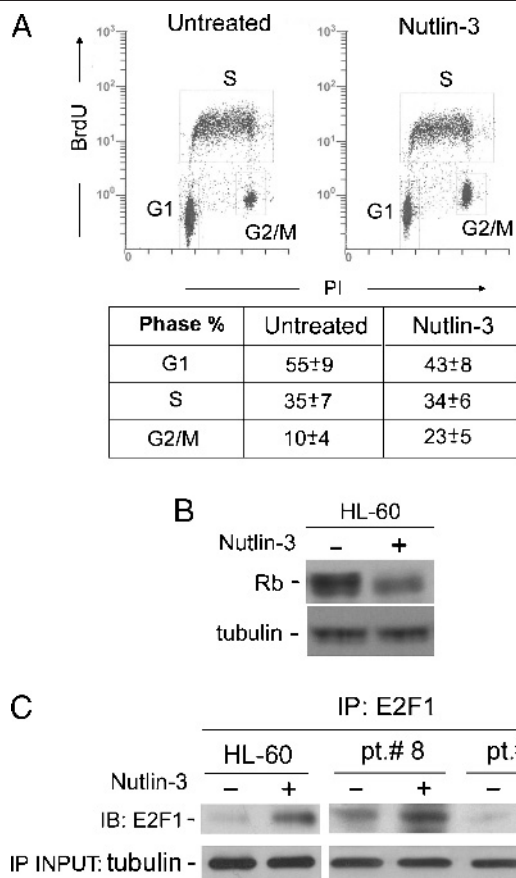
treatment with Nutlin-3 induced the accumulation of E2F1 protein in the  $p53^{-/-}$  HL-60 leukemic cell model. Of note, a similar effect was observed in selected  $p53$  wild-type AML samples, for which a sufficient number of cells were available for the performance of E2F1 immunoprecipitation assay (Figure 4C).

In an attempt to further elucidate the role of E2F1 in AML maturation induced by Nutlin-3, we have used a gene knockdown approach. The transfection procedure adopted in these experiments allowed to efficiently transfect  $40 \pm 5\%$  of HL-60 cells (Figure 5A). HL-60 cells were transfected with a cocktail of two siRNA specific for *E2F1* or with control scrambled siRNA; 48 hours after transfection, cultures were either left untreated or exposed to Nutlin-3. The efficiency and specificity of gene knockdown were documented by the ability of *E2F1* siRNA, but not scrambled siRNA, to re-

duce the induction of E2F1 protein by Nutlin-3 treatment (Figure 5B). In parallel, we could demonstrate that transfection with *E2F1* siRNA, but not with scrambled siRNA, significantly ( $P < .05$ ) counteracted the promaturation activity of Nutlin-3 evaluated in terms of CD14 and CD11b surface expression (Figure 5B).

#### Nutlin-3 Potentiates the Maturation Activity of Recombinant TRAIL

Having previously demonstrated that the TNF family member TRAIL promotes both apoptotic cell death and maturation of the HL-60 myeloid cell line [22], the next experiments were performed to evaluate the effects of the combined treatment Nutlin-3 + TRAIL on both apoptosis and myeloid maturation of HL-60 cells. As shown in Figure 6, A and B, Nutlin-3 significantly ( $P < .05$ ) enhanced the ability of



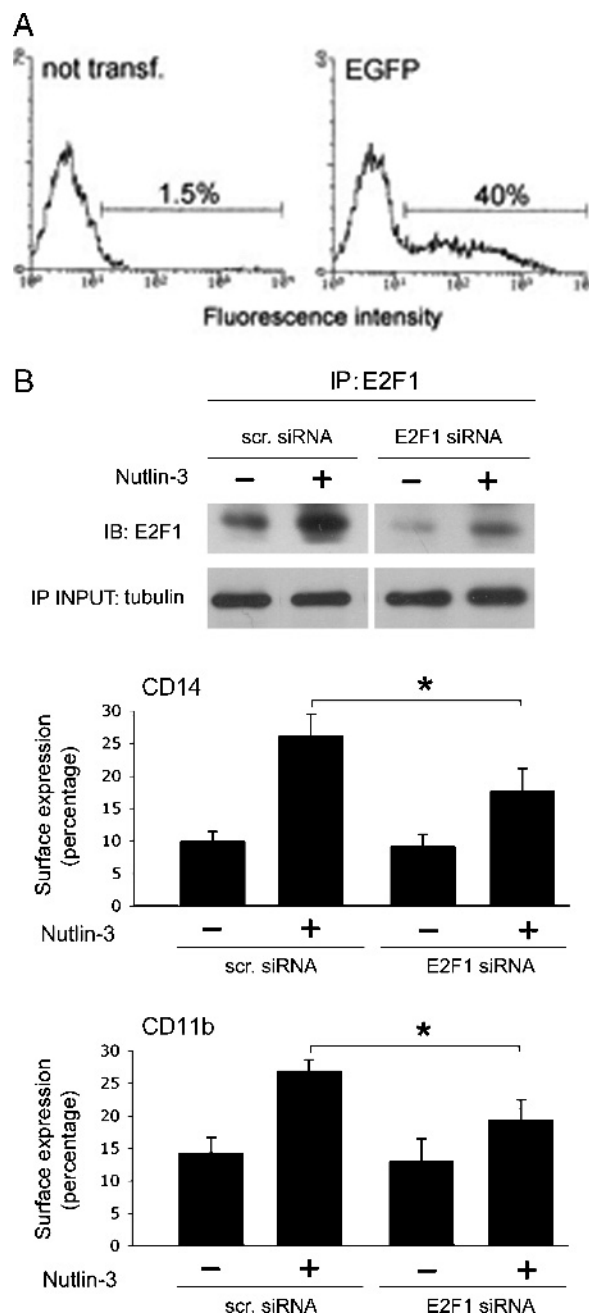
**Figure 4.** Modulation of the cell cycle and induction of E2F1 transcription factor by Nutlin-3. (A) Representative cell-cycle profile of HL-60, either left untreated or treated with Nutlin-3, as analyzed by BrdU/PI staining. The distribution of cells in different phases of the cell cycle was calculated from flow cytograms and expressed as the percentage of the total population. Data are expressed as the mean  $\pm$  SD of the results from six independent experiments. (B) Western blot analysis of total Rb protein levels in HL-60 whole-cell lysates. Tubulin staining is shown as loading control. (C) HL-60 cells and AML samples were treated with Nutlin-3 (10  $\mu$ M) for 24 hours before the analysis of the E2F1 transcription factor by immunoprecipitation (IP) followed by immunoblotting (IB). Tubulin levels of a fraction of the total material used are shown as the control of input lysates.

both TRAIL and TNF- $\alpha$ , the prototype member of the TNF superfamily, to promote the maturation of HL-60 cells. Besides the induction of maturation, it is also noteworthy that TRAIL markedly ( $P < .05$ ) induced the apoptosis of HL-60 cells, whereas neither Nutlin-3 nor TNF- $\alpha$  alone significantly modulated the degree of apoptosis over the background level (Figure 6C). However, although Nutlin-3 did not interfere with the cytotoxic activity of TRAIL, TNF- $\alpha$  significantly ( $P < .05$ ) counteracted the ability of TRAIL to induce apoptosis (Figure 6C).

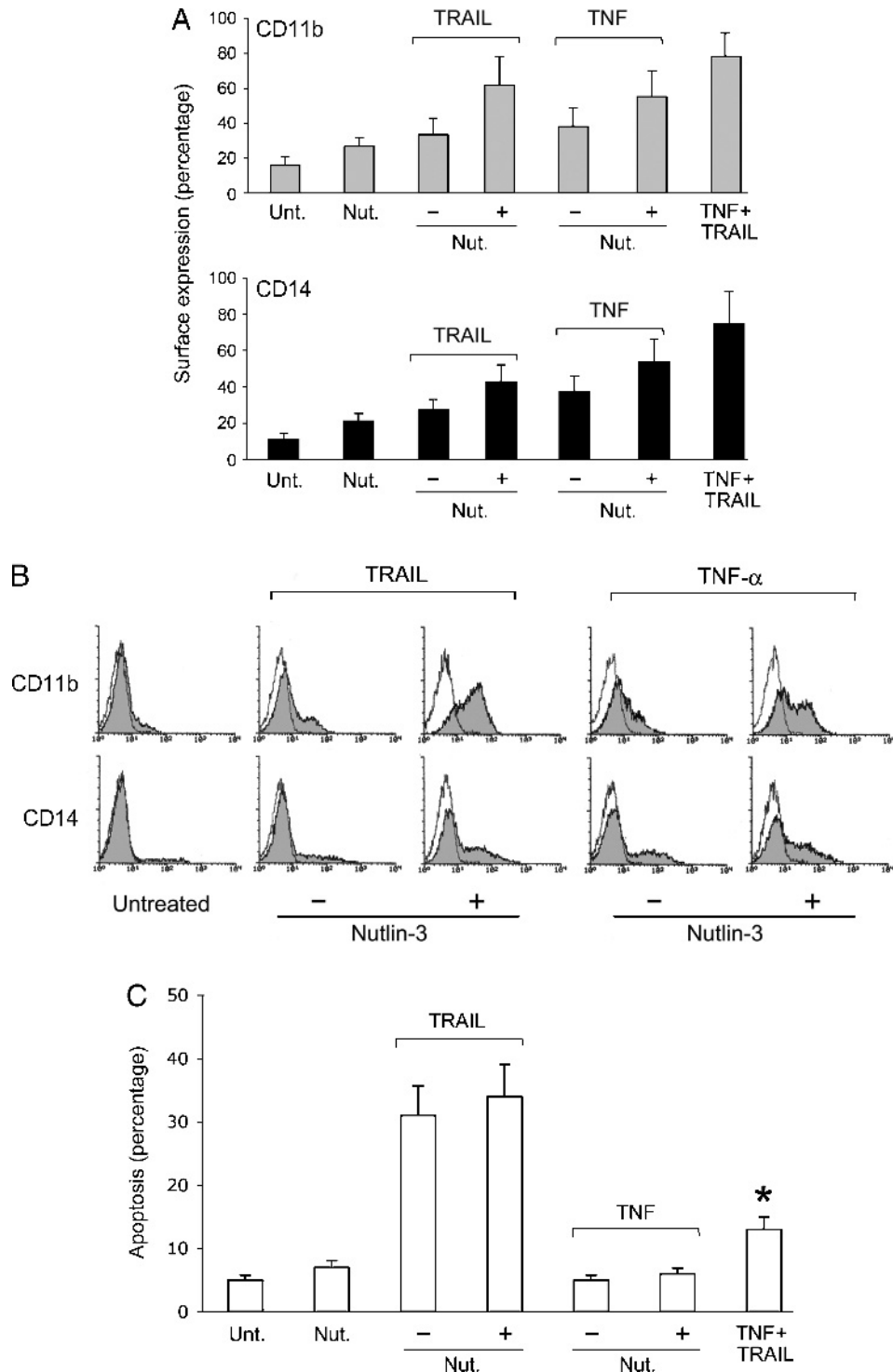
## Discussion

We have here shown for the first time that the small-molecule inhibitor of MDM-2 Nutlin-3 promotes the maturation of both *p53* wild-type and *p53*<sup>-/-</sup> leukemic cells along the myelocytic/monocytic lineage. It is particularly remarkable that such maturational effect was dissociated from the ability of Nutlin-3 to induce apoptosis in AML blasts. It should also be emphasized that although previous studies of our and other groups have

demonstrated the inhibitory effects of Nutlin-3 on the survival of different types of hematologic malignancies, including AML [5–9], this study represents a demonstration of the positive regulative effect of Nutlin-3 on primary AML blasts and HL-60 leukemic cells. Thus, the ability of Nutlin-3 to promote the



**Figure 5.** Nutlin-3 promotes HL-60 maturation through E2F1 induction. HL-60 cells were transfected with control scrambled (scr.) siRNA or E2F1 siRNA and then either left untreated or exposed to Nutlin-3 for 36 hours. In (A), the efficiency of transfection in each experiment was monitored by flow cytometry analyses of EGFP-transfected HL-60 cells. Representative flow cytometry profiles are shown. In (B), the efficiency and specificity of E2F1 knockdown were documented by immunoprecipitation (IP) followed by immunoblotting (IB). Tubulin levels of a fraction of the total material used are shown as the control of input lysates. One of five experiments with similar results is shown. In parallel, surface CD14 and CD11b expression was evaluated by flow cytometry. Data are expressed as the mean  $\pm$  SD of the results from three independent experiments. \* $P < .05$ .



**Figure 6.** Nutlin-3 enhances the maturational activity of both recombinant TRAIL and TNF- $\alpha$  on HL-60 cells. HL-60 cells were left untreated or were treated with TRAIL (0.1  $\mu$ g/ml) and/or TNF- $\alpha$  (10 ng/ml), either alone or in combination with Nutlin-3 (10  $\mu$ M), for 24 hours and then analyzed by flow cytometry for the surface expression of CD11b and CD14 (A and B), as well as for levels of apoptosis (C). In (A), data are expressed as the mean  $\pm$  SD of six independent experiments performed in duplicate. In (B), a representative experiment is shown; shadowed histograms represent cells stained with Abs specific for the indicated surface antigens, whereas unshadowed histograms represent background fluorescence obtained from the staining of the same cells with isotype-matched control Abs. In (C), apoptosis was quantitatively evaluated after Annexin V/PI staining. Data are presented as the mean  $\pm$  SD of six independent experiments performed in duplicate. \* $P < .05$  with respect to treatment with TRAIL alone.

myeloid maturation of both primary AML blasts and HL-60 cells envisions an additional role of Nutlin-3 as an antineoplastic agent, at least for certain types of AML. In fact, induction of mortality by terminal differentiation represents

an alternative approach to the cytodestruction of cancer cells by conventional antineoplastic agents and has important biologic implications. Indeed, it indicates that the malignant state is not an irreversible one and suggests that certain

leukemias may eventually be treated with agents that initiate terminal maturation, presumably with less morbidity than that produced by cytotoxic agents [23]. In this respect, retinoid acids are well-known inducers of the granulocytic differentiation of primary acute promyelocytic leukemia blasts and leukemic cell lines, apparently acting through transcriptional regulation of critical genes [24]. In fact, both retinoids and vitamin D<sub>3</sub> interact with nuclear receptors—members of the steroid/thyroid hormone receptor superfamily of transcription factors [25]. These are ligand-inducible *trans* regulators that modulate the transcription of genes, which play a central role in the control of cell growth and differentiation by interacting with retinoic acid or vitamin D<sub>3</sub> *cis*-acting DNA-responsive elements [24,25]. However, although some retinoids are currently used in the treatment of acute promyelocytic leukemia (the M3 type of AML), agonists such as vitamin D<sub>3</sub> (which are able to induce monocytic differentiation in other subtypes of AML and, in particular, in M4 and M5 types) did not demonstrate efficacy in clinical trials performed in AML patients, mainly due to secondary hypercalcemia, thus limiting the dose of vitamin D<sub>3</sub> that could be administered [26].

Similarly to previous studies [11,12,19], we have also shown that the ability of Nutlin-3 to promote HL-60 maturation was coupled to significant modifications in cell-cycle regulation. However, although a cell-cycle block at the G<sub>0</sub>/G<sub>1</sub> checkpoint has been previously reported [19], our current study demonstrates that Nutlin-3 induced cell-cycle accumulation in G<sub>2</sub>/M. Thus, Nutlin-3 appears to act differently on the cell cycle of leukemic HL-60 cells, as also underlined by the fact that although accumulation of HL-60 cells in G<sub>0</sub>/G<sub>1</sub> was accompanied by accumulation of the hypophosphorylated form of Rb protein [19], Nutlin-3 induced the downregulation of Rb protein in HL-60 cells. In this respect, our findings are in line with a recent study in which a similar downregulation of Rb protein was observed by treatment with Nutlin-3 cell lines derived from solid tumors [13].

It has been shown that the *p53* tumor-suppressor gene is involved in the differentiation of myeloid lineage [27–29] and that overexpression of *p53* in the HL-60 cell line is able to induce differentiation along the granulocytic or monocytic pathway [30,31]. However, these previous data cannot entirely account for our present findings. In fact, we found that Nutlin-3 induces the maturation of both *p53* wild-type and *p53*<sup>-/-</sup> primary AML blasts, as well as of the *p53*<sup>-/-</sup> HL-60 cell line. In an effort to elucidate the molecular mechanism underlining the ability of Nutlin-3 to promote maturation even in *p53*<sup>-/-</sup> AML cells, we could demonstrate that Nutlin-3 induced E2F1 transcription factor, a phenomenon anticipating the differentiation of HL-60 cells. The ability of Nutlin-3 to upregulate E2F1 was in line with the findings of Ambrosini et al. [13], who found that in the *p53*<sup>-/-</sup> HCT116 colon carcinoma cell line, Nutlin-3 disrupted the binding of E2F1 to MDM-2 and induced the transcriptional activation of free E2F1. However, Nutlin-3 induced the degradation of E2F1 in *p53* wild-type cells. The interpretation of these findings was that the loss of E2F1 expression following Nutlin-3 therapy in *p53* wild-type cells appears to be mediated by

increased ubiquitination and subsequent proteasomal degradation, whereas in cells with nonfunctional *p53* (*p53*<sup>-/-</sup> HCT cells), Nutlin-3 decreases the ubiquitination of E2F1, avoiding degradation by the proteasome and making it available for the transcriptional activation of E2F1-related proapoptotic molecules. Taken together, the findings of Ambrosini et al. [13] and our present data suggest that E2F1 might substitute for *p53* in mediating myeloid maturation in *p53*<sup>-/-</sup> AML cells. Moreover, although Ambrosini et al. [13] have described the need for simultaneous treatment with cisplatin to observe an increase in E2F1 in the *p53*<sup>-/-</sup> HCT116 cell line, we have shown that treatment with Nutlin-3 alone was sufficient to induce E2F1 in the HL-60 cell model.

Although the E2F1 transcription factor has been shown to promote either cell proliferation or apoptotic cell death, in relationship with the cell context [30,31], studies performed on *E2F1*<sup>-/-</sup> knockout mice have unequivocally demonstrated the development of a broad spectrum of tumors, suggesting that E2F1 *in vivo* mainly functions as a tumor suppressor. Furthermore, although the overexpression of E2F1 in the *p53*<sup>-/-</sup> M1 myeloblastic leukemia cell lines counteracts IL-6–driven terminal differentiation and maintains leukemogenicity [32], knocked-down mice for E2F transcription factors significantly impaired normal hematopoiesis, with defects at the bone marrow level of both erythroid and myeloid lineages and with a reduced release of mature red blood cells and macrophages [33].

In conclusion, our present study suggests that a therapeutic regimen including Nutlin-3 (possibly in association with recombinant TRAIL) would optimize anticancer activity against leukemic cells by a two-fold mechanism: cytotoxicity in *p53* wild-type cells and induction of differentiation along the monocytic lineage in both *p53* wild-type and *p53*-deleted cells.

## References

- [1] Bueso-Ramos CE, Manshouri T, Haidar MA, Huh YO, Keating MJ, and Albitar M (1995). Multiple patterns of MDM-2 deregulation in human leukemias: implications in leukemogenesis and prognosis. *Leuk Lymphoma* 17, 13–18.
- [2] Moll UM and Petrenko O (2003). The MDM2–*p53* interaction. *Mol Cancer Res* 1, 1001–1008.
- [3] Bond GL, Hu W, and Levine AJ (2005). MDM2 is a central node in the *p53* pathway: 12 years and counting. *Curr Cancer Drug Targets* 5, 3–8.
- [4] Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, Kong N, Kammlott U, Lukacs C, Klein C, et al. (2004). *In vivo* activation of the *p53* pathway by small-molecule antagonists of MDM2. *Science* 303, 844–848.
- [5] Kojima K, Konopleva M, Samudio IJ, Shikami M, Cabreira-Hansen M, McQueen T, Ruvo V, Tsao T, Zeng Z, Vassilev LT, et al. (2005). MDM2 antagonists induce *p53*-dependent apoptosis in AML: implications for leukemia therapy. *Blood* 106, 3150–3159.
- [6] Stuhmer T, Chatterjee M, Hildebrandt M, Herrmann P, Gollasch H, Gerecke C, Theurich S, Cigliano L, Manz RA, Daniel PT, et al. (2005). Nongenotoxic activation of the *p53* pathway as a therapeutic strategy for multiple myeloma. *Blood* 106, 3609–3617.
- [7] Secchiero P, Barbarotto E, Tiribelli M, Zerbini C, di Iasio MG, Gonelli A, Cavazzini F, Campioni D, Fanin R, Cuneo A, et al. (2006). Functional integrity of the *p53*-mediated apoptotic pathway induced by the non-genotoxic agent Nutlin-3a in B-cell chronic lymphocytic leukemia (B-CLL). *Blood* 107, 4122–4129.
- [8] Coll-Mulet L, Iglesias-Serret D, Santidrian AF, Cosials AM, de Frias M, Castano E, Campas C, Barragan M, Fernandez de Sevilla A, Domingo A, et al. (2006). MDM2 antagonists activate *p53* and synergize with genotoxic drugs in B-cell chronic lymphocytic leukemia cells. *Blood* 107, 4109–4114.



- [9] Kojima K, Konopleva M, McQueen T, O'Brien S, Plunkett W, and Andreoff M (2006). Mdm2 inhibitor Nutlin-3a induces p53-mediated apoptosis by transcription-dependent and transcription-independent mechanisms and may overcome Atm-mediated resistance to fludarabine in chronic lymphocytic leukemia. *Blood* **108**, 993–1000.
- [10] Schmitt CA, Fridman JS, Yang M, Lee S, Baranov E, Hoffman RM, and Lowe SW (2002). A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. *Cell* **109**, 335–346.
- [11] Breitman TR, Selonick SE, and Collins SJ (1980). Induction of differentiation of the human promyelocytic leukemia cell line (HL60) by retinoic acid. *Proc Natl Acad Sci USA* **77**, 2936–2940.
- [12] Zauli G, Visani G, Bassini A, Caramelli E, Ottaviani E, Bertolaso L, Bertagnolo V, Borgatti P, and Capitani S (1996). Nuclear translocation of protein kinase C- $\alpha$  and - $\zeta$  isoforms in HL-60 cells induced to differentiate along the granulocytic lineage by all-*trans* retinoic acid. *Br J Haematol* **93**, 542–550.
- [13] Ambrosini G, Sambol EB, Carvajal D, Vassilev LT, Singer S, and Schwartz GK (2007). MDM2 antagonist Nutlin-3a enhances chemotherapy-induced apoptosis in cancer cells with mutant p53 by activating E2F1. *Oncogene* **26**, 3473–3481.
- [14] Secchiero P, Melloni E, Heikinheimo M, Mannisto S, Di Pietro R, Iacone A, and Zauli G (2004). TRAIL regulates normal erythroid maturation through an ERK-dependent pathway. *Blood* **103**, 517–522.
- [15] Secchiero P, Gonelli A, Celeghini C, Mirandola P, Guidotti L, Visani G, Capitani S, and Zauli G (2001). Activation of the nitric oxide synthase pathway represents a key component of tumor necrosis factor–related apoptosis-inducing ligand-mediated cytotoxicity on hematologic malignancies. *Blood* **98**, 2220–2228.
- [16] Brackman D, Lund-Johansen F, and Aarskog D (1995). Expression of leukocyte differentiation antigens during the differentiation of HL-60 cells induced by 1,25-dihydroxyvitamin D<sub>3</sub>: comparison with the maturation of normal monocytic and granulocytic bone marrow cells. *J Leukoc Biol* **58**, 547–555.
- [17] Hmama Z, Nandan D, Sly L, Knutson KL, Herrera-Velitz P, and Reithner NE (1999). 1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub>–induced myeloid cell differentiation is regulated by a vitamin D receptor–phosphatidylinositol 3-kinase signaling complex. *J Exp Med* **190**, 1583–1594.
- [18] Gibellini D, Bassini A, Pierpaoli S, Bertolaso L, Milani D, Capitani S, La Placa M, and Zauli G (1998). Extracellular HIV-1 Tat protein induces the rapid Ser<sup>133</sup> phosphorylation and activation of CREB transcription factor in both Jurkat lymphoblastoid T cells and primary PBMC. *J Immunol* **160**, 3891–3898.
- [19] Seo BR, Lee KW, Ha J, Park HJ, Choi JW, and Lee KT (2004). Saucernetin-7 isolated from *Saururus chinensis* inhibits proliferation of human promyelocytic HL-60 leukemia cells via G<sub>0</sub>/G<sub>1</sub> phase arrest and induction of differentiation. *Carcinogenesis* **25**, 1387–1394.
- [20] Sdek P, Ying H, Zheng H, Margulis A, Tang X, Tian K, and Xiao ZXJ (2004). The central acidic domain of MDM2 is critical in inhibition of retinoblastoma-mediated suppression of E2F and cell growth. *J Biol Chem* **279**, 53317–53322.
- [21] Uchida C, Seiichi Miwa S, Kitagawa K, Hattori T, Isobe T, Otani S, Oda T, Sugimura H, Kamijo T, Ookawa K, et al. (2005). Enhanced Mdm2 activity inhibits pRB function via ubiquitin-dependent degradation. *EMBO J* **24**, 160–169.
- [22] Secchiero P, Gonelli A, Mirandola P, Melloni E, Zamai L, Celeghini C, Milani D, and Zauli G (2002). Tumor necrosis factor–related apoptosis-inducing ligand induces monocytic maturation of leukemic and normal myeloid precursors through a caspase-dependent pathway. *Blood* **100**, 2421–2429.
- [23] Sokoloski JA and Sartorelli AC (1998). Induction of the differentiation of HL-60 promyelocytic leukemia cells by nonsteroidal anti-inflammatory agents in combination with low levels of vitamin D<sub>3</sub>. *Leuk Res* **22**, 153–161.
- [24] Chambon P (1994). The retinoid signaling pathway: molecular and genetic analysis. *Semin Cell Biol* **5**, 115–125.
- [25] Evans RM (1988). The steroid and thyroid hormone receptor superfamily. *Science* **240**, 889–895.
- [26] Bar-Shavit Z, Teitelbaum S, Reitsma SL, Hall P, Pegg A, Trail LG, and Kahn AJ (1983). Induction of monocytic differentiation and bone reabsorption by 1,25-dihydroxyvitamin D<sub>3</sub>. *Proc Natl Acad Sci USA* **80**, 5907–5916.
- [27] Matas D, Milyavsky M, Shats I, Nissim L, Goldfinger N, and Rotter V (2004). p53 is a regulator of macrophage differentiation. *Cell Death Differ* **11**, 458–467.
- [28] Soddu S, Blandino G, Citro G, Scardigli R, Piaggio G, Ferber A, Calabretta B, and Sacchi A (1994). Wild-type p53 gene expression induces granulocytic differentiation of HL-60 cells. *Blood* **83**, 2230–2237.
- [29] Banerjee D, Lenz H, Schnieders B, Manno JD, Ju FJ, Spears CP, Hochhauser D, Danenberg K, Danenberg P, and Bertino RJ (1995). Transfection of wild-type but not mutant p53 induces early monocytic differentiation in HL60 cells and increases their sensitivity to stress. *Cell Growth Differ* **6**, 1405–1413.
- [30] Loughran O and La Thangue NB (2000). Apoptotic and growth-promoting activity of E2F modulated by MDM2. *Mol Cell Biol* **20**, 2186–2197.
- [31] Sdek P, Ying H, Zheng H, Margulis A, Tang X, Tian K, and Xiao ZXJ (2004). The central acidic domain of MDM2 is critical in inhibition of retinoblastoma-mediated suppression of E2F and cell growth. *J Biol Chem* **279**, 53317–53322.
- [32] Amanullah A, Hoffman B, and Liebermann DA (2000). Deregulated E2F-1 blocks terminal differentiation and loss of leukemogenicity of M1 myeloblastic leukemia cells without abrogating induction of p15INK4B and p16INK4A. *Blood* **96**, 475–482.
- [33] Li FX, Zhu JW, Hogan CJ, and DeGregori J (2003). Defective gene expression, S phase progression, and maturation during hematopoiesis in E2F1/E2F2 mutant mice. *Mol Cell Biol* **23**, 3607–3622.